Structural basis for the unusual carbohydrate-binding specificity of jacalin towards galactose and mannose

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Evidence is presented that the specificity of jacalin, the seed lectin from jack fruit (*Artocarpus integrifolia*), is not directed exclusively against the T-antigen disaccharide Gal β 1,3GalNAc, lactose and galactose, but also against mannose and oligomannosides. Biochemical analyses based on surface-plasmon-resonance measurements, combined with the X-ray-crystallographic determination of the structure of a jacalin– α -methyl-mannose complex at 2 Å resolution, demonstrated clearly that jacalin is fully capable of binding mannose. Besides mannose, jacalin also interacts readily with glucose, *N*-acetylneuraminic acid and *N*-acetylmuramic

acid. Structural analyses demonstrated that the relatively large size of the carbohydrate-binding site enables jacalin to accommodate monosaccharides with different hydroxyl conformations and provided unambiguous evidence that the β -prism structure of jacalin is a sufficiently flexible structural scaffold to confer different carbohydrate-binding specificities to a single lectin.

Key words: surface plasmon resonance, X-ray crystallography.

INTRODUCTION

Seeds of jack fruit (*Artocarpus integrifolia*) contain a complex mixture of lectins. Besides the abundant T-antigen-/galactose-binding jacalin [1,2], one minor mannose-binding lectin called KM+, also referred as artocarpin [3,4], has been identified. Jacalin is a homotetrameric protein of four identical protomers consisting of a light (β) and a heavy (α) polypeptide chain of 20 and 133 amino acid residues, respectively [5]. Both chains associate non-covalently into a β -prism monomeric structure $\alpha\beta$ [6]. The structure of mature jacalin is the final result of a complex series of co- and post-translational processing events. Jacalin is synthesized as a preprolectin consisting of a 21-residue signal peptide followed by a 39-residue propeptide, the 20-residue β chain, the linker tetrapeptide Thr-Ser-Ser-Asn and the 133-residue α chain [7]. Co-translational processing involves the removal of the signal peptide and (partial) N-glycosylation, yielding a partly glycosylated prolectin, which is eventually converted into mature jacalin by the excision of the linker tetrapeptide and modification of the N-glycan. During or after processing, four identical protomers form a tetrameric assembly, $\alpha_4\beta_4$ [6].

The carbohydrate-binding specificity of jacalin has been studied intensively since the discovery of its specific interaction with IgA1 [8] and the Thomsen–Friedenreich or T-antigen disaccharide Gal β 1,3GalNAc [9]. Measurements of the intrinsic fluorescence of excited jacalin in the presence of various sugars indicated that D-galactose, β -Met-Gal and 2-deoxy- α -D-galactose are the most potent inhibitors of the lectin [10]. Hapten inhibition of the agglutination of IgA1-coated latex particles by simple sugars and sugar derivatives demonstrated further that the inhi-

bitory activity of galactosides decreases in the following order: *p*-nitrophenyl- α -galactose > α -Met-Gal > melibiose (Gal α 1,6Glc β) > *p*-nitrophenyl- β -galactose > GalNAc > stachyose (Gal α 1,6Gal α 1,6Glc α 1,2Fru β) > galactose > D-galactosamine [11]. In addition, it was observed from the same experiments that α -Met-Man and α -Met-Glc were also weakly inhibitory. Different results were obtained when the specificity was assayed by inhibition of Ig precipitation by jacalin in a gel. In this assay all the sugars tested previously exhibited very comparable inhibitory activities. A comparison of the inhibitory potency of nine sugars on the agglutination of sheep red blood cells or precipitation of human serum protein also yielded some odd results because the order of decreasing inhibitory potency (α -Met-Gal > α -Met-Man = galactose > α -Met-Glc > β -Met-Gal > mannose > fructose > glucose > β -Met-Glc) was difficult to reconcile with results from other experiments [12]. In spite of the obvious inhibition caused by mannose, glucose and their derivatives, jacalin has always been considered a typical galactose-specific lectin. The resolution of the three-dimensional structure of a jacalin– α -Met-Gal complex by X-ray crystallography strengthened this view because the shape of the jacalin carbohydrate-binding site is in good agreement with its presumed galactose-binding specificity [6]. Similar conclusions were drawn with respect to the structure and specificity of the *Machura pomifera* agglutinin (MPA) on the basis of the three-dimensional structure of the lectin complexed with the T-antigen disaccharide Gal β 1,3GalNAc [13]. More recently, Swaminathan et al. [14] reported that the primary binding sites of jacalin and the galactose-specific winged bean lectin exhibit some plasticity. Indeed, both lectins accommodate the bulky 4-*O*-methoxy-galactose derivative through a novel C–H...O type of hydrogen bond [14]. Although these findings

Abbreviations used: Heltuba, *Helianthus tuberosus* agglutinin; HBS, Hepes-buffered saline; MPA, *Machura pomifera* agglutinin; Neu5Ac, *N*-acetylneuraminic acid; MurNAc, *N*-acetylmuramic acid; SPR, surface plasmon resonance.

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have not been interpreted in terms of possible promiscuous specificity, they indicate that the binding sites of these two lectins may accommodate structurally different sugars. If so, the specificity of jacalin should be revisited because some of its biological activities, like strong binding to IgA and strong mitogenicity towards human CD4+ T-lymphocytes (which is exploited as a tool to evaluate the immune status of patients infected with HIV-1) [15], may depend on interactions with carbohydrates other than galactose or the T-antigen. Another reason to re-address the question of jacalin's specificity is the identification of a series of plant lectins, which share a high sequence similarity with jacalin but exhibit a preferential or even exclusive specificity towards mannose or oligomannosides. Examples of these so-called mannose-binding jacalin-related lectins have been identified in *Calystegia sepium* (hedge bindweed), *Helianthus tuberosus* (Jerusalem artichoke), *Musa* sp. (banana), *Oryza sativa* (rice) and jack fruit seeds (KM+). All these lectins are built up of protomers consisting of a single (uncleaved) polypeptide of approx. 150 amino acid residues. Due to the absence of cleavage of the protomer, the mannose-specific jacalin-related lectins possess an extra loop, which makes the binding site inaccessible to galactose.

In this report we present unambiguous evidence based on affinity chromatography and surface-plasmon-resonance (SPR) measurements that jacalin binds not only galactose but also mannose and is fully capable of interacting with high-mannose N-glycans. In addition, it is shown that the binding site of jacalin also accommodates glucose, sialic acid (*N*-acetylneuraminic acid, or Neu5Ac) and *N*-acetylmuramic acid (MurNAc), which demonstrates that jacalin behaves as a 'polyspecific' lectin. Finally, the structural basis for the wide specificity of the jacalin carbohydrate-binding site is demonstrated with the X-ray-crystallographic determination of the structure of a jacalin- α -Met-Man complex at 2 Å resolution.

EXPERIMENTAL

Chemicals

α 1-3, α 1-6 Mannotriose and α 1-3, α 1-6 mannopentaose were purchased from Dextra Laboratories (Reading, U.K.). Asialofetuin, simple and complex sugars, and other chemicals were purchased from Sigma. Arcelin-1 was purified from kidney bean seeds (*Phaseolus vulgaris* L. cv RAZ-2) according to [16]. Seeds were a gift from Dr C. Cardona (Centro Internacional de Agricultura Tropical, Cali, Columbia). Sensor chips (CM 5), Hepes-buffered saline (HBS; 10 mM Hepes/150 mM NaCl, containing 0.05 % surfactant P20 and 3.0 mM EDTA, pH 7.4) and all the chemicals required to activate the CM-dextran and immobilize glycoproteins [100 mM *N*-hydroxysuccinimide, 400 mM *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodi-imide hydrochloride and 1 M ethanolamine hydrochloride (pH 8.5)] were from Pharmacia Biosensor AB (Uppsala, Sweden).

Isolation of jacalin

Jacalin was isolated from jack fruit seeds by affinity chromatography on immobilized galactose. Briefly, dry seeds (20 g) were soaked overnight in distilled water at 2 °C and homogenized in a Waring blender in 200 ml of 0.1 M Tris/HCl (pH 7.5) containing 0.2 M NaCl. The homogenate was centrifuged at 9000 *g* for 15 min and the resulting supernatant filtered through filter paper (Whatman 3MM). The cleared extract was applied to a column (2.5 cm $\times$ 10 cm; approx. 50 ml bed volume) of galactose-Sepharose 4B equilibrated with 0.1 M PBS (pH 7.5). After

loading the extract, the column was washed with the same buffer until the A_{280} value fell below 0.01. The bound jacalin was eluted with a solution of 0.1 M galactose in PBS and dialysed against PBS. To remove any possible trace of KM+, the lectin preparation was rechromatographed on galactose-Sepharose 4B, re-eluted with 0.1 M galactose in PBS, dialysed against the appropriate buffer and stored at -20 °C until use. The crystallographic approach demonstrates clearly that the jacalin preparation was essentially free of KM+.

SPR analysis

The interaction of jacalin with the glycoproteins arcelin-1 and asialofetuin was analysed by SPR using a BIAcore 1000 biosensor (Pharmacia Biosensor AB). For immobilization on CM 5 sensor chips, arcelin-1 and asialofetuin were used at a concentration of 1 mg $\cdot$ ml⁻¹ in 5 mM sodium acetate buffer (pH 4.0). According to the change in SPR response caused by immobilization on the carboxymethylated-dextran layer covering the sensor chip, an estimated surface concentration of 10 ng of immobilized protein/mm² of dextran was obtained. Arcelin-1 contains mainly high-mannose-type N-glycans [16], whereas asialofetuin contains a mixture of N- and O-linked glycans with terminal galactose residues [17,18]. Both proteins possess glycans with a more or less exposed trimannoside core, Man α 1-6(Man α 1-3)Man, which is known to interact specifically with the extended carbohydrate-binding site of many lectins, such as concanavalin A [19].

Jacalin, used at concentrations ranging between 25 and 100 μ g $\cdot$ ml⁻¹ in HBS (pH 7.4), was injected for 5 min on to the glycoprotein-bound surface of the sensor chip at a flow rate of 5 μ l $\cdot$ min⁻¹. The change in the SPR response, expressed in resonance units, was monitored at 25 °C for 9.5 min. The same glycoprotein sensor-chip surface was used repeatedly after removing the remaining bound jacalin by two successive washes with 10 mM HCl and 10 mM NaOH for 2 min each.

Inhibition by sugars was performed by injecting mono-, di- and oligo-saccharides in the concentration range of 5–25 mM in HBS (pH 7.4) at the beginning of the dissociation phase for 5 min at a flow rate of 5 μ l $\cdot$ min⁻¹, and the change in the SPR response monitored at 25 °C for 9.5 min. Inhibition was expressed as the percentage of lectin remaining bound to the glycoprotein-bound surface in the presence of sugar (as compared with measurements performed in the absence of sugars). Results are the means of triplicate experiments.

K_D values of the interaction between jacalin and asialofetuin (exposed galactose residues) or arcelin-1 (exposed mannose residues) were estimated by SPR analysis on a BIAcore 3000 (Pharmacia Biosensor AB). Increasing concentrations of jacalin in HBS (pH 7.4; 3–92 nM) were injected at a flow rate of 30 μ l $\cdot$ min⁻¹ for 5 min on the glycoprotein-bound surface (association phase). HBS was subsequently injected at a flow rate of 30 μ l $\cdot$ min⁻¹ for 4 min (dissociation phase). An estimated surface concentration of 0.3 ng/mm² of dextran was used for both immobilized proteins. K_D values were calculated according to the Langmuir model using BIAevaluation 2.1 software (Pharmacia Biosensor AB).

Crystallization and data collection

For crystallization, jacalin was dialysed extensively against 10 mM Hepes (pH 7.4) and 150 mM NaCl, and concentrated to 18.5 mg $\cdot$ ml⁻¹. Crystals were obtained at 20 °C using the vapour diffusion technique. Typically, 2 μ l of the protein solution were mixed with 2 μ l of the reservoir solution made of 20–22 % poly(ethylene glycol) 8000, 10 % (v/v) isopropanol and 0.1 M

Table 1 Data collection and refinement

$R_{\text{sym}} = |I - \langle I \rangle| / \langle I \rangle$, where I is intensity and $\langle I \rangle$ is the average I value for all observations of equivalent reflections. Values in brackets are for the outer resolution shell. $R_{\text{factor}} = |F_{\text{obs}} - F_{\text{calc}}| / F_{\text{obs}}$. R_{free} is the same as R_{factor} for 2% of the data omitted from the refinement. RMS, root mean square.

	Apo-jacalin	Jacalin- α -Met-Man complex
Resolution (Å)	1.75	1.9
No. of observations	177 742	140 888
No. of unique reflections	54 146	38 468
R_{sym} (%)	3.6 (29.8)	4.2 (12.7)
$I/\sigma(I)$	11.4 (2.1)	9.1 (3.7)
Redundancy	1.8	1.9
Completeness (%)	94.1 (90.1)	98.1 (88.5)
Resolution (Å)	20–1.75	20–2
$R_{\text{factor}} - R_{\text{free}}$ (%)	19.1–22.7	19.1–23.5
No. of reflections (no σ cutoff)	54 082	38 431
RMS deviations		
Bond length (Å)	0.017	0.015
Bond angle (°)	1.78	1.75
Dihedral angle (°)	27.2	27.1
Improper angle (°)	1.1	1.0
Mean B factors (Å ²)		
Main/side chain	24.5/26.5	28.8/30.1
Solvent/sugar	33.5/–	33.8/31.2
RMS deviations on B factors (Å ²)		
Main chain	1.3	1.3
Side chain	2.0	1.9

Hepes (pH 7.5). The jacalin- α -Met-Man complex was obtained after soaking apo-jacalin crystals in the reservoir solution supplemented with 50 mM α -Met-Man (Sigma) for a few hours. Similar soaking experiments were done with Neu5Ac and MurNAc as sugar ligands but the crystals dissolved rapidly when the sugar concentration exceeded 50 mM. Attempts to obtain crystals of jacalin complexed by either Neu5Ac or MurNAc by co-crystallization were also unsuccessful. The jacalin crystals belong to the space group $P2_1$ with the unit cell dimensions $a = 58.8$ Å, $b = 82.3$ Å and $c = 62.9$ Å, and $\beta = 107.6^\circ$, and they contain one jacalin tetramer per asymmetric unit. Crystals selected for data collection were directly flash-cooled at 100 K in a nitrogen gas stream and stored in liquid nitrogen. Data from apo-jacalin and jacalin- α -Met-Man complex were collected on beamlines X11 and BM14 of the Deutscher Elektronen Synchrotron (Hamburg, Germany) and the European Synchrotron Radiation Facility (Grenoble, France), respectively. Oscillation images were integrated with DENZO [20] and scaled and merged with SCALA [21]. Amplitude factors were generated with TRUNCATE [21]. Data-collection statistics and refinements are summarized in Table 1.

Structure determination and refinement

Initial phases were obtained by molecular replacement using the jacalin tetramer (PDB code 1JAC) [6] as a search model with the AMoRe [22], giving a correlation coefficient and an R_{factor} value of 52.2% and 37.9%, respectively, in the 15–4 Å resolution range. Rigid-body refinement was then performed on each subunit with the Crystallography & NMR system (CNS) [23] using data between 20 and 3 Å, and gave an R_{factor} of 24.1%. For 2% of the reflections against which the model was not refined, R_{free} was 24.6%. Refinement of the molecular-replacement model was performed at 1.75 Å resolution using CNS including bulk solvent and anisotropic B-factor corrections and the resulting 2Fo-Fc and Fo-Fc electron-density maps were used to correct

the model with the graphics program TURBO-FRODO [24]. Addition of solvent molecules was performed automatically using CNS and was examined carefully on the graphics display. The final model comprised residues Gly-1 to Leu-133 and Ala-2 to Lys-18 for the α and β chains, respectively. The apo-jacalin structure, without solvent, was used as a starting model for the jacalin- α -Met-Man complex structures. Rigid-body refinement dropped the R_{factor} and R_{free} values to 27% and 28%, respectively, in the 20–3 Å resolution range. Fourier difference maps clearly revealed the location of the bound α -Met-Man in the four subunits, and a final model was obtained after subsequent refinement using CNS, alternated with graphic inspection. A similar procedure was used for the Neu5Ac- and MurNAc-jacalin complexes (results not shown), but in both cases the Fourier difference maps did not reveal the presence of the bound sugar. Inspection of the crystal-packing environment in the jacalin- α -Met-Man complex structure revealed that three out of the four carbohydrate-binding sites in the tetramer are surrounded by symmetry molecules, a feature that could impair binding of bulky sugar molecules, such as Neu5Ac or MurNAc, compared with α -Met-Man. Our model reveals two amino acid differences in the β chain (Lys-6 to Ile and Ser-14 to Pro) compared with the sequence available from the PDB entry 1JAC, an observation in agreement with one of the three SwissProt entries (P18672) reported previously for the jacalin β chain. The tetrameric assemblies of our model and the PDB entry 1JAC are highly similar; the root-mean-square deviations between apo-jacalin and PDB entry 1JAC are 0.35 Å for 133 C α atoms of a single α chain; between apo-jacalin and the jacalin- α -Met-Man complex are 0.29 Å for 133 C α atoms. The stereochemistry of the two models was analysed with PROCHECK [25]; no residues were found in the disallowed regions of the Ramachandran plot. The co-ordinates and structure factors of apo-jacalin and the jacalin- α -Met-Man complex have been deposited with the Protein Data Bank under accession codes 1KU8 and 1KUJ. Figure 3 (see below) was generated with SPOCK [26] and Raster3D [27].

RESULTS

The identification during the last few years of a series of lectins, which share a high sequence similarity with jacalin but exhibit an exclusive specificity towards mannose, raised the question of whether, besides galactose, jacalin also recognizes mannose residues. To address this question, a simple affinity chromatography experiment on immobilized mannose was set up with purified jacalin from jack fruit seeds. Thereby, it was observed that jacalin was retained quantitatively on immobilized mannose (results not shown) and could be eluted with a 0.1 M solution of mannose as well as a 0.1 M solution of galactose. Since the results of these simple experiments indicated strongly that jacalin binds both galactose and mannose, the specificity of the lectin was re-investigated using SPR analysis.

SPR experiments showed that jacalin interacted readily with the complex or *N*-acetyl-lactosaminic-type N- or O-linked glycans of asialofetuin (Figure 1A), as could be expected on the basis of the documented specificity of the lectin towards galactose and the T-antigen. In addition, jacalin also interacted with the high-mannose-type glycans of arcelin-1 (Figure 1B), which confirmed that the lectin recognizes simple sugars as different as galactose and mannose. However, SRP measurements gave K_D values of 3.3×10^9 and 1.3×10^6 M⁻¹ for the interaction of jacalin with asialofetuin (exposed galactose residues) and arcelin-1 (exposed mannose residues), respectively. The unexpected broad specificity of jacalin was corroborated further by hapten-in-

Figure 1 Interaction of jacalin with asialofetuin and arcelin-1

Sensorgrammes showing the interaction in real time of jacalin with asialofetuin (A) and arcelin-1 (B). The upward and downward parts of the graphs correspond to the association (circulating buffer containing jacalin) and the dissociation (circulating buffer devoid of jacalin) phases, respectively. RU, resonance units.

inhibition experiments based on the inhibition by simple sugars or sugar derivatives of the interaction of jacalin with immobilized glycoproteins. As shown in Figure 2, the results of the SPR measurements demonstrated unambiguously that jacalin behaves as a 'polyspecific' lectin, which recognizes the epimeric sugars galactose, mannose and glucose as well as sugar derivatives such as GalNAc, GlcNAc, Neu5Ac, MurNAc and the 1-*O*-methyl derivatives of galactose, glucose and mannose.

The unexpected specificity of jacalin towards both galactose and mannose does not fit the generally accepted idea that this lectin possesses a single binding site with an exclusive specificity towards galactose. In principle, two different explanations are possible: either jacalin possesses two distinct binding sites with a different specificity or the lectin contains a single binding site with a specificity that does not correspond to the presumed galactose-/T-antigen-binding activity. To check the possible occurrence of two distinct carbohydrate-binding sites with different specificities, double binding experiments were performed using immobilized glycoproteins, which contain predominantly either high-mannose-type (arcelin-1) or complex-type (human asialo-serotransferrin) N-glycans. After jacalin was allowed to interact with arcelin-1 bound to a sensor chip, asialotransferrin (at a concentration of $100 \mu\text{g} \cdot \text{ml}^{-1}$) was added during the dissociation phase and the SPR monitored for 30 min. Similarly, arcelin-1 (at a concentration of $100 \mu\text{g} \cdot \text{ml}^{-1}$) was added during the dissociation phase following the binding of jacalin to asialo-serotransferrin immobilized to a sensor chip. In both experiments, no additional increase in SPR was observed, indicating that

Figure 2 Inhibition of jacalin by simple and complex sugars

SPR analysis of jacalin's specificity towards mono- and oligo-saccharides. Top graph: the interaction of jacalin with immobilized asialofetuin in the presence of galactose (◇), α -Met-Gal (○), GalNAc (■), MurNAc (▲) and T-antigen (+). Bottom panel: the interaction of jacalin with immobilized arcelin-1 in the presence of mannose (□), α -Met-Man (●), glucose (×), Neu5Ac (◆), α 1-3, α 1-6 mannotriose (△) and α 1-3, α 1-6 mannopentaose (✕). Sugars were added during the dissociation phase at increasing concentrations ranging from 5 to 25 mM. Results are expressed as the percentage of lectins retained on the immobilized glycoproteins. Values are means from triplicate experiments.

jacalin contains a single carbohydrate-binding site, which interacts with both the high-mannose N-glycans of arcelin-1 and the complex N-glycans of asialotransferrin. This implies that the different sugar-binding specificities must reside in a single carbohydrate-binding site, and that the binding site of jacalin also accommodates mannose and other simple sugars besides galactose.

In a further step towards understanding the carbohydrate polyspecificity of jacalin, we have determined the crystal structures of native jacalin in the absence or presence of bound α -Met-Man and refined them at the respective resolutions of 1.75 and 2.0 Å. The two structures are nearly identical (see the Experimental section) and only the α -Met-Man-bound form will be discussed hereafter.

Our crystallographic studies reveal for the first time a free carbohydrate-binding site for jacalin that allowed us to perform a detailed comparison with the structure of the α -mannose-bound jacalin complex. Whereas no significant differences can be found for most of the C α trace of jacalin, a large movement resides within the carbohydrate-binding site in the surface loop Asn-74-Tyr-78, with the largest difference (1.4 Å) at position Tyr-78. In the apo-jacalin structure, the Tyr-78 side chain is highly disordered and adopts discrete conformations, whereas its side-chain conformation is stabilized and stacked against the B-face of the sugar ring in the α -Met-Man-bound complex (Figure 3A). In addition, three solvent molecules in the apo-jacalin

Figure 3 Quality of the map and carbohydrate-binding site of jacalin

(A) Stereo view of the superposition between apo-jacalin and the jacalin- α -Met-Man complex structure in the vicinity of the carbohydrate-binding site. Movement of the 74–78 surface loop (green) with Tyr-78 and the presence of solvent molecules (blue spheres) are visible in the apo-jacalin structure. The α chains are shown in yellow and orange for apo-jacalin and the jacalin- α -Met-Man complex structure, respectively. The β chains are shown in cyan. (B) Stereo view of the 2.0 Å resolution omit 2Fo-Fc averaged electron-density map, contoured at 1σ , showing bound α -Met-Man and surrounding residues (white carbon, red oxygen and blue nitrogen atoms). The co-ordinates of this region were omitted and the protein co-ordinates were refined by simulated annealing before phase calculation. Hydrogen bonds are shown as dotted lines. (C–E) Molecular surface around the carbohydrate-binding site of jacalin bound to α -Met-Man (C), α -Met-Gal (D) and Heltuba bound to Man α 1-3Man (E), colour-coded yellow and blue for the α and β chains, respectively. The key residues for sugar specificity are shown through a transparent surface; those making van der Waals interactions with the mannose axial O-2 conformation are highlighted in orange. The figure was generated with SPOCK [26] and Raster3D [27].

Table 2 Interactions between carbohydrate ligands and jacalin

The additional van der Waals interactions seen in our structure are shown in *italics*. Numbers in parentheses are those from the PDB accession code 1JAC, corresponding to galactose. – indicates that no bond occurs.

Ligand atom	Protein atom	Distance (Å)
α -Met-Man (Gal) O-6	Asp-125 OD1	2.83 (2.83)
α -Met-Man (Gal) O-6	Trp-123 NH	2.87 (2.92)
α -Met-Man (Gal) O-6	Trp-123 O	3.16 (3.03)
α -Met-Man (Gal) O-6	Tyr-122 NH	2.96 (3.24)
α -Met-Man (Gal) O-5	Tyr-122 NH	3.0 (2.95)
α -Met-Man (Gal) O-3	Gly-1 NH	2.79 (2.76)
α -Met-Man (Gal) O-4	Gly-1 NH	– (3.22)
α -Met-Man (Gal) O-4	Asp-125 OD1	2.82 (2.84)
α -Met-Man (Gal) O-4	Asp-125 OD2	– (3.0)
α -Met-Man (Gal) O-2	Gly-1 C α	3.49 (–)
α -Met-Man (Gal) O-2	Phe-47 CE2	3.37 (4.28)
α -Met-Man (Gal) O-2	Gly-121 C α	3.23 (–)

structure mimic the position of three mannose hydroxyl groups in the α -Met-Man-bound complex, a feature reported frequently for carbohydrate-binding proteins [28,29]. One of these solvent molecules is located at the midpoint that is defined between the axial and equatorial conformation of the sugar hydroxyl O-4 atom of galactose and mannose, respectively, an observation in agreement with our findings showing the plasticity of the jacalin carbohydrate-binding site in the vicinity of the O-4 sugar hydroxyl group.

The bound α -Met-Man is well ordered in all four jacalin subunits present in the crystal structure of the jacalin- α -Met-Man complex (Figure 3B). The mannose-binding site, which occurs at a similar position as that of galactose in jacalin [6] and MPA [13], is located at the centre of three exposed loops

connecting β -strands from Greek keys 2 and 3. Surprisingly, no significant conformational changes are observed within the carbohydrate-binding site in the mannose-bound form of jacalin compared with that bound to galactose, indicating that jacalin can accommodate different sugar molecules without requiring substantial conformational changes, an observation consistent with previous biochemical experiments. The bound mannose establishes similar contacts with jacalin as galactose, except for the O-4 hydroxyl group which establishes only one hydrogen bond with Asp-125 side chain out of the three observed in the jacalin-galactose complex structure (Table 2). Therefore, it appears that the N-terminal amino group of Gly-1 in the α chain, which is not bound to the mannose O-4 equatorial position, is a key residue to dictate the specificity for galactose, as pointed out previously by Sankaranarayanan et al. [6]. However, our structural data reveal that the sugar ring position is shifted by 0.7 Å towards the solvent compared with its position in the galactose-bound form of jacalin, a displacement that may result from the O-2 axial conformation in mannose compared with galactose. Yet, the mannose O-2 hydroxyl group is ideally positioned to establish additional van der Waals contacts with the side chains of Phe-47, Gly-121 and Gly-1 located in the α chain (Figure 3C). These additional interactions compensate partially for the loss of polar interactions at the O-4 hydroxyl group (Figure 3D), and account for the enhanced affinity of jacalin towards galactose-containing glycoproteins (asialofetuin) as compared with high-mannose-containing glycoproteins (arcelin-1). Such difference in the fine sugar specificity, involving only van der Waals contacts, has previously been observed from the structural comparison between the legume LOLI lectin bound to glucose and mannose [30]. Interestingly, both in jacalin and *H. tuberosus* agglutinin (Heltuba), a mannose-binding lectin of the β -prism-fold family, the bound mannose moiety presents a similar hydrogen-bond network (Figure 3E), supporting our observations that jacalin behaves as a mannose-binding lectin.

Figure 4 Shape of the carbohydrate-binding site

Molecular surfaces drawn for jacalin (left-hand panel) and Heltuba (right-hand panel) in complex with α -Met-Man and Man α 1-3Man (white sticks), respectively, showing the more extended area (dark grey) occupied by the carbohydrate-binding site of jacalin compared with Heltuba. The β chain of jacalin is coloured light grey.

Although we failed to obtain crystals of jacalin complexed to other sugar moieties (see the Experimental section), model building with Neu5Ac and MurNAc as ligands, based on the jacalin- α -Met-Man complex structure, indicated that both sugars can readily enter the carbohydrate-binding site of jacalin and anchor to the binding site through a network of hydrogen bonds similar to that occurring in the jacalin-galactose/-mannose complex.

DISCUSSION

A re-investigation of the carbohydrate-binding specificity of jacalin leaves no doubt that the specificity of this lectin is not directed exclusively against galactose but also against mannose and glucose. This implies that there is an important misconception about the specificity of this lectin, which has been known for almost 20 years and, in addition, is used intensively as a specific probe and biologically active protein. Although it is difficult to trace the reasons why the multi-specificity of jacalin has been overlooked, the combined approaches of SRP, X-ray crystallography and molecular modelling revealed that the molecular basis for jacalin's recognition of the epimeric sugars galactose, mannose and glucose resides in some peculiar conformational features of its carbohydrate-binding site. Although unusual, a similar polyspecific character has been reported previously for the cellulose-binding domain CBM13 of the *Streptomyces lividans* xylanase 10A [31] and, most probably, for the lectin isolated from the bitter melon (*Momordica charantia*) [32].

The overall shape of the cleft forming the carbohydrate-binding site is far more extended in jacalin than in the mannose-binding jacalin-related lectins, which exhibit a more restricted specificity for mannose and glucose (Figure 4) [33,34]. As a result, the binding site of jacalin can accommodate epimeric sugars with differently oriented C-2 and C-4 hydroxyl groups and more or less bulky substituents. The presence of an extended site also accounts for the observed specific binding of Neu5Ac and MurNAc to jacalin and for the recently reported binding of 4-Met-Gal to jacalin [14]. Recently, the occurrence of a rather extended carbohydrate-binding site in KM+, the closely related mannose-specific lectin from *A. integrifolia*, was reported from thermodynamic and calorimetric studies of the lectin complexed to a trimannoside [35,36].

Evidently, the discovery of the mannose-binding activity of jacalin also has some practical consequences. First, whenever jacalin is used as a probe to identify galactose residues or T-antigen disaccharide the results should be interpreted with care because mannose or oligomannosides may also be detected. Second, some of the observed biological activities of jacalin may not rely on binding of the lectin to galactose-/T-antigen-containing receptors but on the recognition of receptors with high-mannan glycans. Possibly, the recognition of the multi-specificity of jacalin can help to explain previously poorly understood effects of jacalin [37].

A final remark concerns the high structural similarity between jacalin and MPA [13]. Since the binding sites of both lectins are virtually identical it is likely that the presumed galactose-/GalNAc-specific MPA also possesses extended binding sites that can accommodate mannose and glucose residues.

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